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Cloning and Heterologous Expression of the Phenazine Biosynthetic Locus from *Pseudomonas aureofaciens* 30-84

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Pseudomonas aureofaciens **strain 30-84 suppresses take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Three antibiotics, phenazine-1-carboxylic acid, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine, were responsible for disease suppression. Tn*5*-induced mutants deficient in production of one or more of the antibiotics (Phz$^-$) were significantly less suppressive than the parental strain. Cosmids pLSP259 and pLSP282 from a genomic library of strain 30-84 restored phenazine production and fungal inhibition to 10 different Phz$^-$ mutants. Sequences required for production of the phenazines were localized to a segment of approximately 2.8 kilobases that was present in both cosmids. Expression of this locus in *Escherichia coli* required the introduction of a functional promoter, was orientation-specific, and resulted in the production of all three phenazine antibiotics. These results strongly suggest that the cloned sequences encode a major portion of the phenazine biosynthetic pathway.**

Certain strains of pseudomonads colonize roots and provide biological control of soilborne plant diseases; this results in increased plant health and crop yield (Schroth and Hancock 1981; Suslow 1982; Burr and Caesar 1984; Baker 1985; Schippers *et al.* 1987; Weller 1988). These pseudomonads produce a variety of metabolites, including antibiotics (Leisinger and Margraff 1979; Fravel 1988; Défago and Haas 1990) and pyoverdin siderophores (Baker 1985; Leong 1986; Neilands and Leong 1986; Schippers *et al.* 1987; Loper and Buyer 1991), some of which antagonize fungal pathogens and thereby suppress root disease. Examples of such antibiotics include pyoluteorin and pyrrolnitrin, which are implicated in control of damping-off and wilt diseases of cotton (Howell and Stipanovic 1979, 1980) and vegetable crops (Homma and Suzui 1989; Homma *et al.* 1989; Hasegawa *et al.* 1990) caused by *Pythium ultimum* Trow, *Rhizoctonia solani* Kühn, *Verticillium dahliae* Kleb., and *Fusarium oxysporum* Schlechtend.:Fr.; oomycin A, also suppressive of damping-off of cotton caused by *P. ultimum* (Gutterson 1990; Howie and Suslow 1991); 2,4-diacetylphloroglucinol, involved in control of black root rot of tobacco caused by *Thielaviopsis basicola* (Berk. & Broome) Ferraris and take-all disease of wheat caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker (Keel *et al.* 1990, 1992; Vincent *et al.* 1991); and phenazine-1-carboxylic acid and its derivatives, active in suppression of take-all (Gurusiddaiah *et al.* 1986; Brisbane *et al.* 1987; Brisbane and Rovira 1988; Thomashow and Weller 1988) and speckled blotch of wheat caused by *Septoria tritici* Roberge in Desmaz. (Flaishman *et al.* 1990).

Phenazine-1-carboxylic acid (PCA) has a dominant role in the control of take-all by *Pseudomonas fluorescens* (Trevisan) Migula 2-79. Strains that produce the antibiotic (Phz$^+$) are significantly more protective of wheat, as indicated by reduced disease severity (Thomashow and Weller 1988; Hamdan *et al.* 1991; Ownley *et al.* 1992) and lesion number (Bull *et al.* 1991), than are phenazine-deficient (Phz$^-$) mutant strains. In contrast, production of the pyoverdin siderophore and an additional antifungal factor, Aff, contribute little, if any, to suppressiveness (Hamdan *et al.* 1991; Ownley *et al.* 1992). Protection by strain 2-79 occurs over a range of soil pH from 4.9 to 8.0 (Ownley *et al.* 1992), varies in response to soil composition (Ownley *et al.* 1990), and is correlated directly with production of the antibiotic *in situ* (Thomashow *et al.* 1990). Production of the antibiotic also may be a factor in ecological fitness; populations of Phz$^-$ mutants decline more rapidly over time in natural environments than do those of Phz$^+$ strains (Mazzola *et al.* 1990).

More than 50 different naturally occurring phenazine compounds are produced by *Pseudomonas* and *Streptomyces* spp. All are pigmented, and many exhibit broad-spectrum antibiotic activity against bacteria and fungi as well as some higher plants and animals (Toohey *et al.* 1965b; reviewed in Turner and Messenger 1986). Proposed modes of action include DNA intercalation and the inhibition of RNA synthesis (Turner and Messenger 1986) and, because of their ability to undergo oxidation-reduction reactions, the generation of cytotoxic superoxides and peroxides (Hassan and Fridovich 1980) or the disruption of energy-requiring, membrane-associated metabolic processes such as active transport (Baron *et al.* 1989).

Phenazines are products of the shikimic acid pathway; chorismate is the probable branchpoint intermediate, and the amide group of glutamine is the primary source of ring nitrogen. The identity of intermediates between choris-

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mic acid and the first-formed phenazine, probably phenazine-1,6,-dicarboxylic acid, is still unclear. Anthranilic acid has frequently been considered an attractive candidate, but most studies do not support its role as a precursor (Turner and Messenger 1986). In *P. aeruginosa* (Schroeter) Migula PAO1, however, a novel anthranilate synthase that appears to function specifically in the synthesis of pyocyanine, the blue phenazine pigment characteristic of the strain, was recently identified (Essar *et al.* 1990). This enzyme is distinct from the *trpEG*-encoded anthranilate synthase associated with tryptophan biosynthesis and is encoded by a separate locus designated *phnAB*. Whether *phnAB* homologues are present or participate in the production of anthranilate for phenazine synthesis in other pseudomonads is not known.

Our attention initially was drawn to *P. aureofaciens* Kluyver 30-84 by its brilliant orange pigmentation, which is characteristic of the species (Kluyver 1956; Haynes *et al.* 1956). Strains of *P. aureofaciens* produce mainly PCA and 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA), the latter being responsible for the color (Kluyver 1956; Haynes *et al.* 1956; Olson and Richards 1967; Toohey *et al.* 1965a), but at least six other phenazine derivatives also have been reported (Turner and Messenger 1986). Strain 30-84 suppresses take-all (D. M. Weller and R. J. Cook, personal communication), and we were intrigued that it and strain 2-79 might share biosynthetic capability for a class of antibiotics that has limited natural distribution. We initiated the present study, therefore, to determine the significance of antibiotic(s) in biological control with strain 30-84 and to identify the gene(s) required for antibiotic production. Phenazine compounds were isolated and identified, and mutants deficient or altered in pigmentation were generated and compared to the wild type for suppressive activity against take-all. Detailed analysis of one locus resulted in identification of genes distinct from *phnAB* that encode a major portion of the phenazine biosynthetic pathway.

MATERIALS AND METHODS

Bacteria, fungi, and plasmids. Bacteria, fungi, and plasmids used in this work are described in Table 1. *P. aureofaciens* strain 30-84 was isolated from the roots of wheat taken from a site in north central Kansas, where wheat had been grown for 65 consecutive years and the incidence of take-all had declined spontaneously (W. W. Bockus, personal communication). A spontaneous, rifampicin-resistant mutant was selected and used interchangeably with the wild type in this study. Colonies typically were orange, exhibited orange fluorescence under longwave (365 nm) UV irradiation, and were surrounded by a dark halo of UV absorbance consistent with production of PCA (Thomashow and Weller 1988). Strain 30-84 or its derivatives were grown at 28° C in nutrient broth yeast extract (NBY) (Vidaver 1967), pigment production medium (PPM) (Levitch and Stadtman 1964), AB minimal medium (Schleif and Wensink 1981), Kanner minimal medium supplemented with one-fifth strength potato extract (KMPE) (Thomashow and Weller 1988), or King's medium B (KMB) (King *et al.* 1954). *Escherichia coli* (Migula) Castellani and Chalmers strains were grown at 37° C in Luria-Bertani medium (LB) containing 5 g/L of NaCl (Maniatis *et al.* 1982) except

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[y]	Source or reference
Pseudomonas aureofaciens		
30-84	Phz$^+$, Rif^r	W. W. Bockus
Escherichia coli		
S17-1	*thi pro hsdR hsdM recA rpsL* RP4-2 (Tc^r::Mu) (Km^r::Tn*7*)	Simon *et al.* 1983
DH5α	F$^-$ *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*argF-lacZYA*) *U169* ϕ80d*lacZ*Δ*M15* λ$^-$	Bethesda Research Laboratories (BRL)[z]
HB101	F$^-$ *hsdS20* (r^-_B,m^-_B) *supE44 recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-5* λ$^-$	BRL
JM-105	*endA1 thi rpsL sbcB15 hsdR4* Δ(*lac-proAB*) (F^r *traD36 pro lacI*^{q} ZΔ*M15*)	Yanish-Perron *et al.* 1985
Plasmids		
pSUP1021	pACYC184::Tn*5* Tc^r Mob$^+$	Simon *et al.* 1986
pLAFR3	IncP Tc^r *cos*$^+$ *rlx*$^+$	Staskawicz *et al.* 1987
pRK2013	IncP Km^r TraRK2$^+$ *repRK2* repEl$^+$	Ditta *et al.* 1980
pBR325	ColEI Cm^r Tc^r	Boliver *et al.* 1978
pUC18	ColEI Ap^r	Yanisch-Perron *et al.* 1983
pRZ102	ColEI Tn*5*	Jorgensen *et al.* 1979
pLSP259	pLAFR3 containing 30-84 genomic DNA	This study
pLSP282	pLAFR3 containing 30-84 genomic DNA	This study
pLSP28-11::Tn*5*	30-84 Chromosomal DNA with Tn*5* insert	This study
pLSP57-30::Tn*5*	30-84 Chromosomal DNA with Tn*5* insert	This study
pLSP259::Tn*5*-3 through pLSP259::Tn*5*-46	Tn*5* inserts into pLSP259	This study
pLSP20-2-1	pBR325::9.2-kb *Eco*RI fragment	This study
pLSP20-2-1REV	Reverse orientation of 9.2-kb *Eco*RI fragment	This study
pLSP18-6	pUC18::9.2-kb *Eco*RI fragment	This study
pLSP18-6H3Δ2	Deleted to *Hin*dIII site 3.8 kb from 3′ end	This study
pLSP18-6SmΔ9-22	Deleted to *Sma*I site 4.9 kb from 3′ end	This study
pLSP18-6SmΔ15-5	Deleted to *Sma*I site 5.6 kb from 3′ end	This study

[y] Ap^r, Cm^r, Rif^r, and Tc^r indicate resistance to ampicillin, chloramphenicol, rifampicin, and tetracycline, respectively. Phz$^+$, produces all phenazines at wild-type levels.
[z] Gaithersburg, MD.

for strain JM105, which was grown in M9 minimal medium (Maniatis *et al.* 1982) containing 0.2% maltose and 1 mg of thiamine hydrochloride per liter. Cultures of *G. g.* var. *tritici* were maintained on one-fifth strength potato-dextrose agar (PDA) (Thomashow and Weller 1988), and inoculum for plant assays was prepared as described (Wilkinson *et al.* 1985). Antibiotics used included kanamycin sulfate (50 μg/ml), chloramphenicol (100 μg/ml), and rifampicin (75 μg/ml). Tetracycline was used at 25 μg/ml except in mating selection plates, where it was used at 40 μg/ml.

Antibiotic isolation and chromatography. Phenazine antibiotics were isolated from cultures of strain 30-84 in PPM broth shaken at room temperature for 3–5 days. Cultures were acidified to pH 2.0 with concentrated HCl, and phenazines were extracted into benzene and concentrated by evaporation as described previously (Thomashow *et al.* 1990). Samples were fractionated on thin-layer silica G chromatography plates in an ascending benzene/acetic acid solvent mixture (95:5 by volume). Individual phenazine pigments were visualized by UV irradiation at 365 nm. Removal of the cells by centrifugation before acidification had no apparent effect on antibiotic yield. Phenazine spots were eluted from thin-layer plates and fractionated by high-performance liquid chromatography (HPLC) (Waters Associates, Inc., Milford, MA) with a Nova-Pak 4μ C_{18} reverse-phase radial pack cartridge and a linear gradient of 0–100% acetonitrile in water. Both solvents contained 0.1% trifluoroacetic acid. Individual phenazine peaks were collected, and spectra were analyzed with a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). Because it was difficult to resolve mixtures of PCA and 2-OH-PCA by thin-layer chromatography (TLC) or HPLC, the two compounds were first separated by multiple partitioning between benzene and 0.1 N NaOH (adapted from Toohey *et al.* 1965a). Each antibiotic then migrated as a single homogeneous peak.

Tn*5* mutagenesis and mutant characterization. Tn*5* insertions were generated in strain 30-84 by conjugation with *E. coli* S17-1(pSUP1021) (Simon *et al.* 1983, 1986) at a donor/recipient ratio of 1:1. Recipient cultures were shaken overnight at room temperature in NBY containing 100 μg/ml of iron ammonium citrate, adjusted to 1×10^9 cells per milliliter, and 50-μl samples were spotted onto sterile nitrocellulose filters on prewarmed NBY agar supplemented as above with iron. Donor cultures were grown overnight at 37° C in LB broth containing tetracycline. Before mating, the cultures were centrifuged at 5,000 × *g* for 5 min and suspended to 1×10^9 cells per milliliter in fresh LB. Samples (50 μl) were spotted onto filters containing the recipient cells, and the plates were incubated at 28° C for 24–48 hr. The filters were then transferred to 2 ml of sterile water, and samples (0.2 ml) of the bacterial suspension were spread on NBY plates supplemented with rifampicin, kanamycin, and tetracycline. After incubation for 4–5 days, exconjugants were patched onto AB minimal, KMB, and PPM plates. Nutritional requirements were determined as described elsewhere (Lederberg 1950). Pyoverdin siderophore mutants were identified by nonfluorescence under longwave UV irradiation on KMB medium. Phz^- mutants were identified initially by alteration or loss of colony pigmentation on PPM agar under visible or longwave UV irradiation. Changes in phenazine production were confirmed by TLC and HPLC. Phz^- mutants also were tested for production of hydrogen cyanide on KMB agar containing 4.4 g/L of glycine. A strip of Cyantesmo HCN indicator paper (Gallard-Schlesinger Industries, Inc., Carle Place, NY) was taped inside the lid of the plate. The plates were sealed with Parafilm and incubated at 28° C for 24–48 hr. A positive test was indicated by a change in the paper from colorless to blue. Strain 30-84 and *P. fluorescens* 2-79, which does not produce detectable HCN, were included as positive and negative controls, respectively.

DNA manipulation and library construction. Genomic and plasmid DNAs were prepared as described previously (Thomashow and Weller 1988). Transformation, Southern transfer and hybridization, nick translation, and agarose gel electrophoresis were performed essentially as described by Maniatis *et al.* (1982). We constructed a genomic library of strain 30-84 DNA by ligating partially digested *Eco*RI fragments of 25–35 kilobases (kb) into the *Eco*RI site of cosmid vector pLAFR3 (Staskawicz *et al.* 1987). Ligated DNA was packaged with Packagene (Promega Biotec, Madison, WI) and transduced into *E. coli* JM105. Transductants containing recombinant plasmids were identified by tetracycline resistance and white colony color on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside and the inducer isopropyl-β-D-thiogalactopyranoside. The presence of inserts was confirmed by restriction enzyme analysis. The library consisted of 1,152 individual clones that were stored in 40% glycerol at −20° C in microtiter plates.

Complementation of Phz^- mutants. Individual cosmids from the genomic library were introduced into Phz^- mutants by a modification of the triparental mating procedure described previously (Thomashow and Weller 1988). Recipient cultures were grown overnight in NBY agar containing 100 μM iron ammonium citrate and kanamycin, centrifuged (5,000 × *g* for 5 min), mixed in a 1:1 ratio with overnight cultures of *E. coli* HB101(pRK2013) (Ditta *et al.* 1980), and spread onto NBY agar containing iron. The genomic library was replicated onto fresh LB plates containing tetracycline, grown overnight at 37° C, and replicated again onto plates spread with the helper-recipient mixture. The plates were incubated at 28° C for 48 hr and replicated onto NBY agar containing rifampicin, kanamycin, and tetracycline. We identified transconjugants restored to phenazine production after 4–5 days by scoring visually for orange pigmentation and UV absorption. We retested single donors by mating them on filters to confirm complementation.

Wild-type sequences from complementing cosmids were recombined into the chromosomes of Phz^- mutants by marker exchange. The vector pLAFR3 is unstable in *P. aureofaciens* 30-84 and its derivatives without direct selection for tetracycline resistance (Tc^r) (data not shown). However, double recombination can occur between the homologous plasmid-borne wild-type sequences and the chromosomal Tn*5*-containing mutated sequences; this results in the integration of a single, stable copy of the wild-type region in the genome. The frequency with which such double recombinants were recovered varied with the

sizes of the flanking regions of homology. Cultures of Phz$^-$ mutants of strain 30-84 containing complementing cosmids were grown in LB, transferred daily three to five times, and plated on LB agar. Pigmented colonies were subsequently screened for sensitivity to kanamycin and tetracycline (Km^s and Tc^s). Loss of the vector was confirmed by Southern hybridization.

Tn*5* mutagenesis of pLSP259. Tn*5* insertions were introduced into cosmid pLSP259 with a defective λ::Tn*5* phage (deBruijn and Lupski 1984). Samples (0.25 ml) from a culture of DH5α(pLSP259) grown overnight without aeration in LB containing tetracycline and 0.2% maltose were dispensed into multiple tubes, each containing 25 μl of a suspension of λ::Tn*5* (initial concentration of 1×10^{10} pfu/ml). After 5–20 min at room temperature, 4.5 ml of LB supplemented with kanamycin and tetracycline was added, and the tubes were incubated at 28° C for 36–48 hr. Plasmid DNA was isolated from each culture and transformed into DH5α. We analyzed plasmid DNA prepared from individual Tc^r, Km^r transformants by restriction enzyme digestion to localize sites of Tn*5* insertion.

Deletion analysis of the 9.2-kb *Eco*RI fragment. Plasmid pLSP18-6 was digested partially with *Hin*dIII, *Pst*I, *Sal*I, *Sph*I, or *Sma*I. Each digest was ligated and transformed into DH5α. Tc^r transformants were scored for orange fluorescence and UV absorption, and the resulting plasmid deletion derivatives were analyzed by agarose gel electrophoresis.

Fungal inhibition *in vitro*. Four samples (5 μl), two each from overnight cultures of wild-type 30-84 and a Phz$^-$ mutant strain, were spotted near the perimeter of a KMPE agar plate such that the two wild-type (control) samples were opposite each other and perpendicular to the mutant samples. After incubation for 24–48 hr at 28° C, a 0.5-cm plug of *G. g.* var. *tritici* from the leading edge of a culture grown for 5–6 days was placed in the center of the plate. We determined the size of the zone of fungal inhibition 4–5 days later by measuring the distance (in millimeters) between the edge of the bacterial colonies and the leading edge of fungal hyphae. Inhibition was expressed as the ratio of the average distance between the edge of the fungus and the mutant colonies divided by the average distance between the edge of the fungus and the wild-type colonies. Assays were repeated at least twice and usually replicated five times.

Take-all suppression assay. Suppression of take-all was determined with wheat seedlings grown in plastic tubes as described previously (Thomashow and Weller 1988). Two bacteria-treated seeds (1×10^5–5×10^6 cfu/seed) were sown in Cone-Tainers (16.5 × 2.5 cm top diameter) containing 10 g of steamed (100° C, 1 hr) Palouse silt loam soil amended with 0.45% (w/w) oat kernel inoculum of *G. g.* var. *tritici*. The seeds were covered with 1 cm of sterile vermiculite, maintained in a growth chamber (15° C, 12-hr light cycle), and watered twice weekly with one-third strength Hoagland's solution (macroelements only). Seedlings were removed from the tubes after 21–28 days, and the roots were washed. Plant height (in centimeters) was measured, and root disease was rated on a scale of 0–8 (0 indicating a healthy plant and 8, a dead plant) as previously described (Thomashow and Weller 1988). Treatments were arranged in a randomized complete block design, and each treatment was replicated five times. Seeds treated with only methylcellulose were included as a control. An analysis of variance and Fisher's protected LSD test ($P < 0.05$) (Statistical Analysis System, SAS version 6.06, SAS Institute, Cary, NC) were used to identify statistically significant differences among treatments.

RESULTS

Characterization of phenazine antibiotics produced by strain 30-84. Three phenazine antibiotic spots were detected in extracts from cultures of strain 30-84 analyzed by TLC. The first, compound I, absorbed UV light and migrated with the same R_f (0.17) as PCA purified from *P. fluorescens* 2-79 (Table 2). Compound I tentatively was identified as PCA according to its comigration with purified PCA, its crystalline appearance as long, thin yellow needles, and its positive sodium hydrosulfite (specific for carboxyl groups) and negative formalin (specific for a hydroxyl group at the number two position of the benzene ring)

Table 2. Relative mobilities and UV-visible absorption maxima of phenazine antibiotics produced by *Pseudomonas aureofaciens* 30-84 and *Escherichia coli* DH5α(pLSP18-6)[u]

Compound[w]	R_f	Absorption maxima (nm)[v]		
		0.1 N NaOH	$CHCl_3$	30% CH_3CN
P. aureofaciens 30-84				
PCA	0.17	250(250)[x] 366(364)	252(252)[y], 370, (369)	250, 372
2-OH-PCA	0.19	230(230)[x], 282(285), 367(367), 489(488)	236, 256, 360, 390	258, 368, 446
2-OH-PZ	0.04	226(229)[z], 275(275), 367(367), 475	234, 256, 360, 388	..., 258, 388, 438
E. coli DH4α(pLSP18-6)				
PCA	0.17	252, 368		248, 372
2-OH-PCA	0.19	248, 282, 362, 484		248, 368, 428
2-OH-PZ	0.04	218, 256, 367, 484		236, 255, 375, 488

[u] Cultures (20 ml) in LB or LB + ampicillin were shaken at 28° C for 24–48 hr. Phenazines were extracted into acidified benzene and separated by thin-layer chromatography on silica gel G in benzene/acetic acid (95:5).
[v] Average values from two to five separate determinations. Values in $CHCl_3$ are for samples purified by thin-layer chromatography; values in NaOH and in 30% CH_3CN and 0.1% trifluoroacetic acid are for samples separated by high-performance liquid chromatography.
[w] PCA, phenazine-1-carboxylic acid; 2-OH-PCA, 2-hydroxyphenazine-1-carboxylic acid; 2-OH-PZ, 2-hydroxyphenazine.
[x] Values in parentheses are averages of values reported by Gerber (1973), Toohey *et al.* (1965a), and Gurusiddaiah *et al.* (1986).
[y] Average of values reported by Gerber (1973) and Chang and Blackwood (1969).
[z] Values reported by Levitch and Rietz (1966).

reactions (Gerber 1973). Compound II, the second phenazine spot, migrated similarly to PCA (R_f of 0.19) but fluoresced orange under UV light. It tentatively was identified as 2-OH-PCA by its crystalline appearance as orange rods and plates and its positive formalin and sodium hydrosulfite reactions. Compound III, a comparatively minor spot, also fluoresced orange under UV light but migrated with an R_f of 0.04 and was tentatively identified as 2-hydroxyphenazine (2-OH-PZ) according to its crystalline appearance as orange rods and plates and its positive formalin and negative sodium hydrosulfite reactions. It produced an orange-red solution in alkali that became red-violet with the addition of ammonia and formalin; this is consistent with the properties of 2-OH-PZ rather than of 1-hydroxyphenazine (Mann 1970).

Each compound was purified by HPLC, and its UV-visible absorption spectrum was determined (Fig. 1). Spectra for compounds I, II, and III closely matched those reported (Toohey *et al.* 1965a; Levitch and Reitz 1966; Gerber 1973) for PCA, 2-OH-PCA, and 2-OH-PZ, respectively (Table 2).

Isolation of Phz$^-$ mutants. Km^r exconjugants of strain 30-84 were recovered at a frequency of 5.2×10^{-6} per initial recipient after mutagenesis with Tn*5*. Efficient mutagenesis required that recipient cultures be pregrown and conjugated in the presence of either 100 μg/ml of iron ammonium citrate or 0.01 M *p*-aminobenzoic acid. Without these additives, which appear to suppress phenazine synthesis, frequencies of Km^r exconjugants declined to below 10^{-8}.

Approximately 21,000 Km^r exconjugants from 13 independent matings were screened. Of these, 1.0% were auxotrophic, with a variety of nutritional requirements; 0.5% were defective in pyoverdin production; and approximately 0.4% were Phz$^-$ as judged initially by loss or alteration of colony pigmentation. Phz$^-$ mutants were unimpaired in production of both pyoverdin and hydrogen cyanide, as indicated by colony fluorescence on KMB agar under longwave UV light and by a positive result with an HCN-specific indicator. No plasmid DNA was detected in strain 30-84 by standard or megaplasmid isolation procedures (D. Heron, L. S. Pierson, and L. S. Thomashow, unpublished), suggesting that all Tn*5* insertions, including those implicated in phenazine production, are chromosomal. The frequency of Phz$^-$ mutants recovered is consistent with involvement of a limited number of loci in antibiotic production.

Identification of a phenazine biosynthetic locus. Segments of DNA containing sequences required for phenazine production were isolated by two different procedures. First, we screened cosmids from a genomic library of wild-type 30-84 DNA to identify those that could complement Phz$^-$ mutants to Phz$^+$. Although each of 10 cosmids complemented one or more of 10 different strains that were completely deficient in phenazine production, only two restored all 10 mutants to phenazine production. These cosmids, pLSP259 and pLSP282, contained identical *Eco*RI fragments of 11.2 kb and 9.2 kb, and pLSP282 contained an additional 3.7-kb *Eco*RI fragment (Fig. 2).

Southern hybridization with the Tn*5*-specific probe pRZ102 indicated that genomic DNA from individual Phz$^-$ mutants contained single transposon insertions into at least

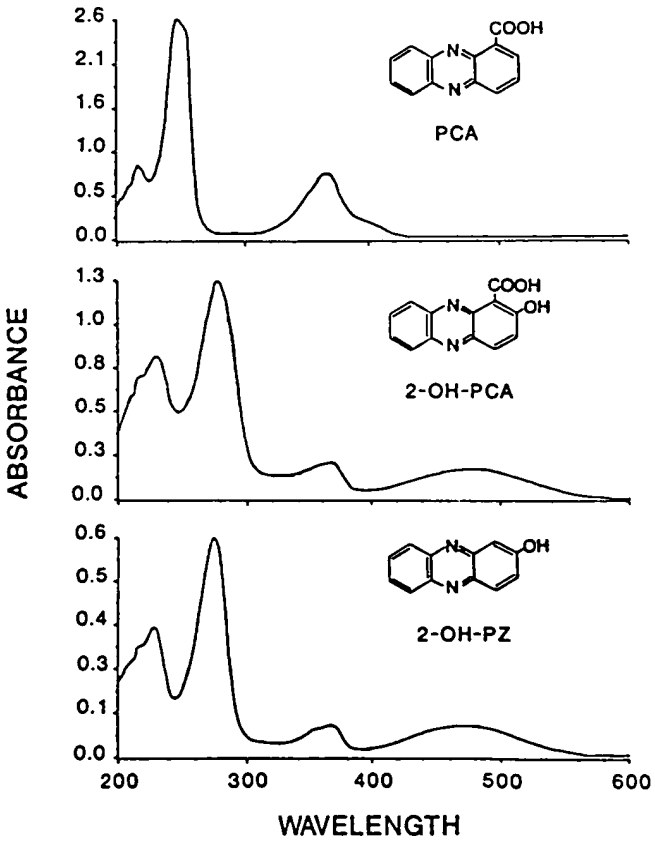

Fig. 1. Ultraviolet-visible spectra and structures of phenazine antibiotics produced by *Pseudomonas aureofaciens* 30-84. Spectra were determined in 0.1 N NaOH from compounds purified by high-performance liquid chromatography. The chemical structure of each phenazine compound is adjacent to its corresponding spectrum. PCA, phenazine-1-carboxylic acid; 2-OH-PCA, 2-hydroxyphenazine-1-carboxylic acid; 2-OH-PZ, 2-hydroxyphenazine.

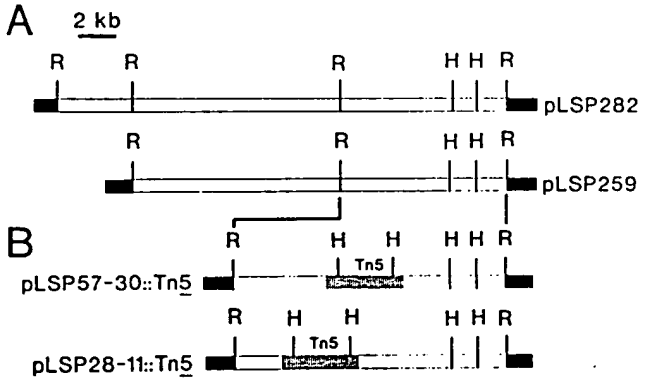

Fig. 2. Physical maps of plasmids containing a phenazine biosynthetic region from strain 30-84 of *Pseudomonas aureofaciens* and Tn*5* insertions that disrupt phenazine production. A, Overlapping cosmids pLSP259 and pLSP282. Open lines indicate chromosomal DNA from strain 30-84; solid lines indicate pLAFR3 DNA. B, Tn*5* insertions and flanking sequences cloned from phenazine mutants. Open lines indicate cloned, mutated sequences; cross-hatched lines indicate Tn*5* sequences; solid lines indicate pBR325 DNA. Strain 30-84.57-30, from which pLSP57-30::Tn*5* was obtained, produced phenazine-1-carboxylic acid. Strain 30-84.28-11, the source of pLSP28-11::Tn*5*, produced no phenazine antibiotics. R, *Eco*RI; H, *Hin*dIII.

five different *Eco*RI restriction fragments (data not shown). As a second approach to identifying loci required for phenazine production, we cloned fragments containing Tn*5* from two mutants, 30-84.28-11 and 30-84.57-30, into the unique *Eco*RI site in plasmid pBR325 by selecting for the kanamycin resistance determinant within the transposon. Because Tn*5* has no *Eco*RI sites, each insert contained the transposon plus flanking chromosomal DNA extending to the nearest *Eco*RI site on either side. The flanking regions from two such recombinant plasmids, pLSP28-11::Tn*5* and pLSP57-30::Tn*5*, hybridized strongly with the 9.2-kb *Eco*RI fragment present in cosmids pLSP259 and pLSP282. The physical maps of the cloned DNA in these plasmids and in the cosmids are identical when the size of the Tn*5* insertion element is taken into account (Fig. 2).

The mutant strains from which pLSP28-11::Tn*5* and pLSP57-30::Tn*5* were derived differed markedly in phenotype. Strain 30-84.28-11, the source of pLSP28-11::Tn*5*, was totally deficient in phenazine production, whereas 30-84.57-30, from which pLSP57-30::Tn*5* was cloned, was yellow and produced PCA but not 2-OH-PCA or 2-OH-PZ. Thus, insertion of Tn*5* into contiguous regions of the 30-84 chromosome in strains 30-84.28-11 and 30-84.57.30, respectively, inactivated linked loci required for the production of PCA and for the conversion of PCA to its 2-hydroxyphenazine derivatives.

Localization of phenazine biosynthetic regions in pLSP259. The phenazine biosynthetic locus was further delineated by mutagenesis of pLSP259 with Tn*5*. Seventeen independent insertion derivatives of the cosmid were introduced individually by triparental mating into the Phz$^-$ mutants 30-84.28-11 and 30-84.44-8. Three classes of insertion derivatives that differed in ability to complement the two mutants to Phz$^+$ *in trans* were recovered (Fig. 3A). The first group had no effect on complementation and presumably included insertions outside the region required for phenazine synthesis. The second group allowed complementation for production of PCA but not for the 2-hydroxyphenazine derivatives, suggesting inactivation of sequences involved specifically in phenazine hydroxylation. The third class rendered pLSP259 completely unable to complement for phenazine production, which was consistent with insertion into sequences essential for the synthesis of all phenazines. Collectively, these insertions identified approximately 2.8 kb within the 9.2-kb *Eco*RI fragment as necessary for production of the 2-hydroxyphenazine derivatives, of which approximately 1.9 kb was required for the synthesis of PCA. Three additional Tn*5* insertions within the larger 11.2-kb *Eco*RI fragment of pLSP259 also inactivated the ability to complement for production of the 2-hydroxyphenazine derivatives.

Production of phenazine antibiotics in *E. coli*. Colonies of *E. coli* DH5α containing pLSP259 were white and produced no detectable phenazine antibiotics. However, approximately 50% of transformants containing the subcloned 9.2-kb *Eco*RI fragment from pLSP259 (Fig. 2)

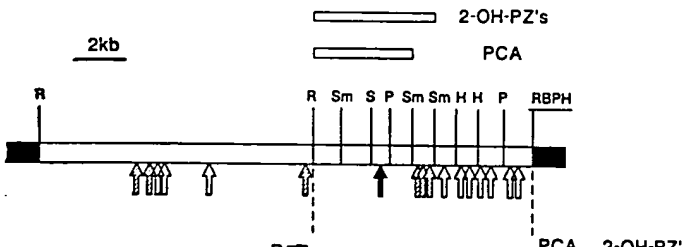

Fig. 3. Localization of the phenazine biosynthetic region by Tn*5* mutagenesis of pLSP259 and deletion analysis of pLSP18-6. A, pLSP259::Tn*5* insertion derivatives and their ability to complement for phenazine production. Open arrows indicate sites of insertion that have no effect on complementation; cross-hatched arrows indicate insertions that complement for production of phenazine-1-carboxylic acid (PCA) only; solid arrows indicate an insertion that blocks complementation for all phenazine production. Open lines indicate DNA from strain 30-84 of *Pseudomonas aureofaciens*; solid lines indicate pLAFR3 DNA. Open bars above the map indicate the 1.9- and 2.8-kb regions involved in the production of PCA and the 2-hydroxyphenazine derivatives, respectively. B, Deletion derivatives of pLSP18-6. Open lines indicate 30-84 DNA; cross-hatched lines indicate pUC18 DNA. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I.

Table 3. Inhibition *in vitro* of *Gaeumannomyces graminis* var. *tritici* by Phz$^-$ mutant and restored strains of *Pseudomonas aureofaciens* 30-84[u]

Strain	Relevant characteristics[v]	Relative inhibition[w]
30-84	Phz$^+$, Rif^r, orange	1.00 ± 0.00
30-84.RRR6	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.QQQ1	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.A21	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.L14	30-84::Tn*5* Phz$^-$, white	0[x]
30-84.CCC46	30-84::Tn*5* Phz$^-$, white	0.01 ± 0.01
30-84.33-45	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.40-30	30-84::Tn*5* Phz$^-$, white	0[x]
30-84.41-6	30-84::Tn*5* Phz$^-$, white	0.01[x]
30-84.44-27	30-84::Tn*5* Phz$^-$, white	0.05[x]
30-84.23-39	30-84::Tn*5* Phz$^-$, white	0.10[x]
30-84.28-11	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.44-8	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.GG46	30-84::Tn*5* Phz$^-$, yellow-white	0.23 ± 0.07
30-84.43-26	30-84::Tn*5* Phz$^-$, yellow-orange	0.40[x]
30-84.57-30	30-84::Tn*5* Phz$^-$, tan-yellow[y]	0.46 ± 0.11
30-84.61-48	30-84::Tn*5* Phz$^-$, yellow-orange[z]	0.75 ± 0.06
30-84.28-11	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.28-11R	30-84.28-11 Phz$^+$, recombinant, orange	1.06 ± 0.03
30-84.44-8	30-84::Tn*5* Phz$^-$, white	0.00 ± 0.00
30-84.44-8R	30-84.44-8 Phz$^+$, recombinant, orange	0.95 ± 0.10

[u] Inhibition was determined on Kanner minimal medium supplemented with one-fifth strength potato extract (Thomashow and Weller 1988). Plugs of *G. g.* var. *tritici* were added two days after the bacteria, and plates were measured 4–5 days later.

[v] Rif^r, resistant to rifampicin; Phz$^+$, produces all phenazines at wild-type levels; Phz$^-$, deficient or altered in phenazine production.

[w] Average values of two to six separate determinations unless otherwise indicated. Inhibition is expressed as the ratio of the average distance from the edge of the fungus to the mutant or restored colony divided by the average distance from the fungus to a wild-type colony on the same plate. In each experiment, assays were replicated at least twice and usually five times.

[x] Results of a single determination.

[y] Mutant 30-84.57-30 produced mainly phenazine-1-carboxylic acid.

[z] Mutant 30-84.61-48 produced relatively more of the 2-hydroxyphenazines than the wild type.

in pBR325 appeared orange and were surrounded by a dark halo under UV irradiation. Pigmentation was dependent on the orientation of the 9.2-kb fragment, suggesting that transcription in *E. coli* initiated within the vector, presumably from the chloramphenicol promoter. Orientation-dependent pigmentation also occurred in colonies of *E. coli* containing pLSP18-6, in which the 9.2-kb *Eco*RI fragment was located downstream of the *lac* promoter in pUC18 (Fig. 3A). Extracts of pigmented cultures contained compounds that comigrated by TLC and were visually indistinguishable by UV irradiation from PCA, 2-OH-PCA, and 2-OH-PZ purified from strain 30-84. After separation by HPLC, these compounds had the same retention times as the phenazines but differed somewhat in their spectra (Table 2), probably because of interference from other compounds in the extracts from *E. coli*.

Deletion derivatives of pLSP18-6. A series of deletion derivatives from the putative 3′ end of the 9.2-kb *Eco*RI fragment was constructed in pLSP18-6, in which expression of the phenazine locus in *E. coli* is driven by the *lac* promoter of pUC18 (Fig. 3B). Deletion to a *Hin*dIII site 3.8 kb from the 3′ end had no effect on phenazine production in *E. coli*. Deletion to a *Sma*I site approximately 4.9 kb from the 3′ end allowed continued production of PCA but not of the two hydroxyphenazine derivatives, and deletion to a second *Sma*I site approximately 5.6 kb from the 3′ end eliminated all phenazine production. These results localize the biosynthetic region within 3.6 kb of the *Eco*RI site adjacent to the *lac* promoter and are consistent with results obtained by Tn*5* insertional inactivation of cosmid pLSP259.

Fungal inhibition and suppression of take-all. All mutants of strain 30-84 that were totally deficient in production of the three phenazine antibiotics failed to inhibit growth of *G. g.* var. *tritici* on KMPE agar (Table 3). Other mutants that were quantitatively or qualitatively altered in phenazine production, according to colony pigmentation, exhibited reduced levels of inhibition. For example, the bright red mutant 30-84.61-48 was 70–80% as inhibitory as the orange wild type. The yellow mutant 30-84.57-30, shown by TLC to produce PCA but not the 2-hydroxyphenazine derivatives, inhibited *G. g.* var. *tritici* at approximately 50% of the wild-type level.

Phz$^-$ mutants also were significantly less suppressive of take-all than wild-type 30-84 (Table 4). In most cases, plants from seed treated with mutants such as 30-84.44-8, which produced no phenazines, had as much disease as plants that received no bacterial treatment. In contrast, plants from seed treated with mutants such as 30-84.57-30 or 30-84.61-48, which were altered in phenazine production, had more take-all than those treated with wild-type 30-84, but less than those treated with phenazine-deficient mutants or with methylcellulose alone.

To confirm the importance of the phenazine antibiotics in antifungal activity, we reintroduced wild-type sequences from cosmid pLSP259 into the chromosomes of Phz$^-$ mutants 30-84.28-11 and 30-84.44-8 by marker exchange. Strains 30-84.28-11R and 30-84.44-8R, in which the Tn*5*-containing chromosomal regions were replaced by wild-type sequences from cosmid pLSP259, were Km^s and Tc^s and exhibited wild-type colony pigmentation and inhibition both *in vitro* and in the take-all suppression assay (Tables 3,4). Loss of the vector was confirmed by Southern hybridization (data not shown).

DISCUSSION

This study demonstrates that production of the phenazine antibiotics PCA, 2-OH-PCA, and 2-OH-PZ is the primary mechanism by which *P. aureofaciens* 30-34 suppresses take-all of wheat. Loss of production of the three phenazines eliminated all, or nearly all, of the suppressive activity of the strain, and restoration of the ability to produce the antibiotics concomitantly restored suppressive activity. The importance of the hydroxyphenazines, as well as PCA, in suppression by strain 30-84 is clearly indicated by the diminished inhibitory capabilities of mutant 30-84.57-30, which produced only PCA, and mutant 30-84.61-48, which was altered in pigmentation and presumably in hydroxyphenazine content (Tables 3,4). Phenazine-deficient mu-

Table 4. Suppression of take-all by phenazine-producing and nonproducing strains of *Pseudomonas aureofaciens* 30-84[x]

Strain	Phz $^{+/-}$	Experiment								
		1	2	3	4	5	6	7	8	9
30-84	+	4.86 a	4.16 b	2.96 a	5.75 a	1.63 a	2.35 a	3.95 a	2.03 a	3.67 a
30-84.RRR6	−	5.47 bc					4.48 f		6.78 d	
30-84.QQQ1	−						4.48 f		6.77 d	
30-84.GG46	−	6.14 cd	6.54 d	6.32 c						
30-84.A21	−	5.87 d								
30-84.CCC46	−	5.78 cd								
30-84.Z7	−	6.38 d								
30-84.28-11	−			2.83 a	6.45 cd	6.29 d	4.32 de	6.96 e	6.75 d	7.11 e
30-84.44-8	−			7.09 d	6.75 d	6.03 d	4.51 f	7.06 e	6.93 de	7.20 e
30-84.57-30	+[y]		3.46 a				2.75 b	4.60 b	2.78 b	
30-84.61-48	+[z]		4.74 c				2.76 b	5.27 c	2.60 b	
30-84.28-11R	+				5.96 ab	5.66 c				4.33 c
30-84.44-8R	+				5.83 a	1.86 a		3.89 a	6.92 de	3.37 a
Control (no bacteria)		6.16 d	7.26 e	7.28 d	6.38 c	6.37 d	4.58 f	7.14 e	7.09 e	7.30 e

[x] Wheat seed treated with bacteria or with methylcellulose alone was sown in steamed soil infested with *G. g.* var. *tritici*. After 3–4 wk of incubation, the seedlings were washed free of soil and evaluated for disease severity on a scale of 0 (healthy)–8 (dead). Means in the same column followed by the same letter do not differ by least significant difference analysis ($P < 0.05$).

[y] Produces mainly phenazine-1-carboxylic acid.

[z] Produces mainly 2-hydroxyphenazines.

tants continued to produce a pyoverdin siderophore and hydrogen cyanide but were at most only slightly more protective of wheat than the control treatment, suggesting that neither metabolite has a major role in the suppression of take-all by the bacteria. Similarly, neither pyoverdin (Hamdan *et al.* 1991; Ownley *et al.* 1992) nor HCN (Haas *et al.* 1991) was a major factor in the control of take-all by *P. fluorescens* strains 2-79, M4-80R, and CHA0. Siderophores and HCN might, however, contribute to the overall competitiveness of these strains in the rhizosphere (Weller 1988).

With earlier work on strain 2-79 (Thomashow and Weller 1988; Thomashow *et al.* 1990), the results presented here demonstrate that production of phenazine antibiotics is required for maximum suppression of take-all by at least two different species of root-colonizing fluorescent pseudomonads. Production of phenazine compounds by another species, *P. aeruginosa* LEC1, recently was shown to be important in the suppression of speckled blotch, a foliar disease of wheat caused by *Septoria tritici* (Flaishman *et al.* 1990). The phenazines, therefore, represent one class of antibiotics that can, when produced in sufficient quantity by introduced biocontrol agents, contribute to the control of plant diseases. Other such classes of antibiotics include pyoluteorin (Howell and Stipanovic 1980), pyrrolnitrin (Howell and Stipanovic 1979; Homma and Suzui 1989; Hasegawa *et al.* 1990), and derivatives of acetylphloroglucinol (Keel *et al.* 1990, 1992; Vincent *et al.* 1991). Current evidence supports the idea that antibiosis is the dominant mechanism of disease suppression by introduced strains that produce these substances. However, some phenazines and acetylphloroglucinols can adversely affect plant metabolism (Toohey *et al.* 1965b; Haas *et al.* 1991; Keel *et al.* 1992), suggesting that they also might have the potential to stimulate defense mechanisms, including induced resistance, in host plants.

Hydroxyphenazine compounds are thought to be derived sequentially from PCA (Flood *et al.* 1972; Römer and Lange 1983) and are responsible for the characteristic orange pigmentation of 30-84 and other *P. aureofaciens* strains (Kluyver 1956; Haynes *et al.* 1956). The production of PCA, 2-OH-PCA, and 2-OH-PZ by cultures of *E. coli* containing a 9.2-kb *Eco*RI fragment from strain 30-84 strongly suggests that the cloned sequences encode a structural locus for a major portion of the phenazine biosynthetic pathway. Mutagenesis and deletion analyses identified a segment of approximately 2.8 kb that was essential for production of all three of the phenazines, of which 0.9 kb was required specifically for hydroxyphenazine synthesis in strain 30-84 and in *E. coli* (Fig. 3). Therefore, the enzymes catalyzing the formation of PCA and the hydroxyphenazine derivatives would be encoded, respectively, within contiguous 1.9- and 0.9-kb segments. These sequences alone apparently are not sufficient for production of the hydroxyphenazines in strain 30-84, however, because three independent Tn*5* insertions into the 11.2-kb *Eco*RI fragment bordering the biosynthetic region in pLSP259 eliminated the ability of the cosmid to complement Phz$^-$ mutants for hydroxyphenazine production (Fig. 3). One of these insertions was immediately proximal to the biosynthetic region in the cosmid and may have influenced its expression in mutants of strain 30-84. Whether the region defined by the other two insertions, which are 8–9 kb further upstream, is functionally replaced by *E. coli* genes or is not required in *E. coli* in the absence of the native promoter is presently unknown. In strain 30-84, the inability of the chromosome to complement these upstream insertions may be due to the titration of limited quantities of an activator by multiple copies of a target sequence present *in trans* on the cosmid. It is also unclear if the biosynthetic locus encodes a mechanism of resistance to the phenazines. Phenazine antibiotics are toxic to *E. coli* (Toohey *et al.* 1965b; Thomashow and Weller 1988), and it was necessary to establish conditions that suppressed phenazine production by strain 30-84 before matings with *E. coli* donor strains could be performed successfully. It is interesting that *E. coli* strains in which the phenazine biosynthetic locus was expressed were able to remain viable. Phz$^-$ mutants of strain 30-84 also appeared fully resistant to the antibiotics produced by the wild-type strain (L. S. Pierson, unpublished).

Cultures of *E. coli* containing pLSP259 did not produce phenazine antibiotics, indicating that the native promoter does not function in the heterologous host. Expression of the biosynthetic locus in *E. coli* required introduction of an alternate promoter and was orientation-sensitive, indicating direction of transcription within the cloned region. The endogenous phenazine promoter presumably is located near the end of the 11.2-kb *Eco*RI fragment of pLSP259 bordering the phenazine biosynthetic genes (Fig. 2). Many pseudomonad promoters, especially those involved in secondary or unique metabolic pathways, are not recognized in *E. coli* because of lack of either a specific alternate sigma factor or another ancillary positive-activating factor (Hedges *et al.* 1977; Deretic *et al.* 1987). Genes encoding such factors may be linked to the biosynthetic locus itself, as is the case for oomycin A, an antibiotic produced by the biocontrol strain *Pseudomonas* sp. Hv37a (Gutterson 1990), or they may be present in some of the other, apparently unlinked, loci that were identified by Tn*5* mutagenesis as essential for phenazine production in strain 30-84. Clearly, however, the regulatory mechanisms that govern the expression of the phenazine locus in strain 30-84 can be circumvented in *E. coli* by introduction of a readily expressed promoter upstream of the 2.8-kb biosynthetic sequences.

Whether anthranilate, synthesized via the *trpEG* locus, serves as a precursor for the synthesis of the phenazines in *E. coli* is not known. However, the 9.2-kb fragment lacked significant homology, even at low stringency, with *phnAB*, which is the anthranilate synthase locus associated with phenazine synthesis in *P. aeruginosa* (Essar *et al.* 1990; L. S. Thomashow, L. S. Pierson, and D. W. Essar, unpublished). These results suggest either that *E. coli* can provide substrate for phenazine production in the form of anthranilate or some similar metabolite or that substrate may arise via a different pathway than the one proposed for *P. aeruginosa*. Enzymes encoded within the 1.9-kb segment would then be responsible for assembly of the phenazine ring and conversion of hypothetical phenazine intermediates, such as phenazine-1,6-dicarboxylic acid, to PCA. Additional enzyme(s) required for the formation of 2-OH-PCA and 2-OH-PZ probably are encoded within the 0.9-

kb segment. This scheme is consistent with a proposed pathway in which 2-OH-PCA and 2-OH-PZ are sequentially derived from PCA, perhaps via an arene oxide intermediate, in a reaction catalyzed by a mixed function oxidase (Flood *et al.* 1972). Further characterization of the biosynthetic locus from strain 30-84 undoubtedly will shed light on the mechanism of ring assembly, which is still unknown, and on the subsequent derivatization reactions.

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